Comparative metagenomics reveals a diverse range of antimicrobial resistance genes in effluents entering a river catchment

ARTICLE *in* WATER SCIENCE & TECHNOLOGY · DECEMBER 2015

Impact Factor: 1.11 · DOI: 10.2166/wst.2015.634

6 AUTHORS, INCLUDING:

David W Verner-Jeffreys

Centre for Environment, Fisheries and Aquac…

59 PUBLICATIONS **612** CITATIONS

SEE PROFILE

Craig Baker-Austin

Centre for Environment, Fisheries and Aquac…

66 PUBLICATIONS **1,526** CITATIONS

SEE PROFILE

Gareth Peter Pearce

University of Cambridge

54 PUBLICATIONS **1,040** CITATIONS

SEE PROFILE

Comparative metagenomics reveals a diverse range of antimicrobial resistance genes in effluents entering a river catchment

Will Rowe, David W. Verner-Jeffreys, Craig Baker-Austin, Jim J. Ryan, Duncan J. Maskell and Gareth P. Pearce

ABSTRACT

The aquatic environment has been implicated as a reservoir for antimicrobial resistance genes (ARGs). In order to identify sources that are contributing to these gene reservoirs, it is crucial to assess effluents that are entering the aquatic environment. Here we describe a metagenomic assessment for two types of effluent entering a river catchment. We investigated the diversity and abundance of resistance genes, mobile genetic elements (MGEs) and pathogenic bacteria. Findings were normalised to a background sample of river source water. Our results show that effluent contributed an array of genes to the river catchment, the most abundant being tetracycline resistance genes *tetC* and *tetW* from farm effluents and the sulfonamide resistance gene *sul2* from wastewater treatment plant (WWTP) effluents. In nine separate samples taken across three years we found 53 different genes conferring resistance to 7 classes of antimicrobial. Compared to the background sample taken up river from effluent entry, the average abundance of genes was three times greater in the farm effluent and two times greater in the WWTP effluent. We conclude that effluents disperse ARGs, MGEs and pathogenic bacteria within a river catchment, thereby contributing to environmental reservoirs of ARGs.

Key words | antimicrobial resistance, aquatic environment, metagenomics, resistome

Will Rowe (corresponding author)
Duncan J. Maskell
Gareth P. Pearce
Department of Veterinary Medicine,
University of Cambridge,
Cambridge,
UK
E-mail: *wpmr2@cam.ac.uk*

David W. Verner-Jeffreys
Craig Baker-Austin
Centre for Environment,
Fisheries and Aquaculture Science,
Weymouth,
UK

Jim J. Ryan
Environment,
Health and Safety, GlaxoSmithKline,
Ware,
UK

LIST OF ABBREVIATIONS

ARG	Antimicrobial Resistance Gene
MGE	Mobile Genetic Element
WWTP	Wastewater treatment plant
SEAR	Search Engine for Antimicrobial Resistance
RPMK	Reads Per Kilobase per Million
BWA	Burrows-Wheeler Aligner
RAC	Repository of Antibiotic resistance Cassettes

INTRODUCTION

Antimicrobial resistance remains a significant and growing concern for both human and veterinary clinical practice (Levy & Marshall 2004, Davies & Davies 2010), with infections that were once readily treated now being resilient to antimicrobial therapy (WHO 2012). The use of antimicrobial compounds exerts selection pressures on bacteria, leading to the fixation of gene mutations, selection of resistant precursors and the up-regulation and lateral transfer of antimicrobial resistance genes (ARGs) within prokaryotic communities (Gillings 2013). The maintenance and transfer of ARGs is responsible in part for the rising threat of antimicrobial resistance (Laxminarayan *et al.* 2013).

The collective pool of ARGs in a given environment is termed the resistome (D'Costa *et al.* 2006, Wright 2007). Although a proportion of these ARGs are genes that have evolved to utilise antimicrobial compounds for functions other than defence, such as signalling molecules or constituents of metabolic pathways (Linares *et al.* 2006, Dantas *et al.* 2008), the resistome may also serve as a reservoir for ARGs that can be transferred to clinically significant pathogens (Forsberg *et al.* 2012, Wellington *et al.* 2013). Indeed, ARGs are commonly associated with mobile genetic elements (MGEs) that facilitate the transfer of ARGs between bacteria

doi: 10.2166/wst.2015.634

and enable their entry into the accessory genome of pathogenic bacteria (William *et al.* 2013).

There is growing evidence showing that aquatic environments harbour ARGs, MGEs and pathogenic bacteria (Chen *et al.* 2013, Lu *et al.* 2015, Devarajan *et al.* 2015). It is also likely that these environments may host many uncharacterised and novel ARGs that may be selected for under sufficient selection pressures (Bengtsson-Palme *et al.* 2014). Effluents that feed into the aquatic environment have also been shown to contain ARGs, such as the effluents of urban residential areas and hospitals (Li *et al.* 2015), as well as other wastewater and faecal sources (Pruden *et al.* 2006, Zhang *et al.* 2009, Li *et al.* 2012) but the abundance and diversity of these genes relative to background samples needs to be clarified. It is therefore crucial to establish whether effluents entering the aquatic environment are carrying ARGs, along with MGEs and pathogenic bacteria, thus contributing to the reservoirs of resistance genes that may be utilised by pathogenic bacteria and subsequently re-enter human and animal populations (Berendonk *et al.* 2015).

Previous studies into the presence of ARGs within the aquatic environment have utilised techniques such as bacterial culture and polymerase chain reaction (Tao *et al.* 2010; Zhang & Zhang 2011; Lu *et al.* 2015). These techniques offer the ability to detect phenotypic resistance (culture), or a panel of ARGs, but they are limited by culturing bias or inadequate detection panels. Next generation sequencing techniques, such as metagenomics, offer the ability to circumvent these limitations and identify all known ARGs within a sample (if suitable reference sequences are available), providing a new approach for the environmental monitoring of antibiotic resistance (Port *et al.* 2014).

In this study we have identified two distinct effluents that enter a single river catchment. Both effluents originate from faecal sources and were sampled several times, immediately prior to them entering the environment. Using a comparative metagenomic approach, we describe the ARG content of these effluents, characterise the MGEs and pathogenic bacteria present, and relate the abundance of these features to a background sample of the river source water, taken from upstream of the effluent entry points.

METHODS

Sample collection and DNA sequencing

Water samples were collected from three sources within the River Cam Catchment, Cambridge, UK. A pilot collection was made on 21st June 2012 (Rowe *et al.* 2015). Further collections were made on the 2nd May 2013 and 4th August 2014. The effluent of the municipal wastewater treatment plant (WWTP) (latitude: 52.234469, longitude: 0.154614) was collected annually from the treated effluent discharge pipe that enters the River Cam. The effluent of the University of Cambridge dairy farm (latitude: 52.22259, longitude: 0.02603) was collected annually prior to it being applied to the surrounding fields as fertiliser, where it subsequently enters drainage ditches that drain into the River Cam. The river source water of the River Cam was collected at Ashwell Spring (latitude: 52.0421, longitude: 0.1497) once on the 4th August 2014. Samples were collected in 10L sterile polypropylene containers, transported at 4 °C to the laboratory and processed within 2 hours.

Sample filtration, metagenomic DNA extraction and sequencing

Similarly as in Dancer *et al.* (2014), samples were filtered under pressure at approximately 2 bar using a pressure vessel system (10 L SM 1753, Sartorius). Samples were first pre-filtered through 3.0 μm membranes (Millipore) at 2 Bar to remove eukaryotic cells and debris. The filtrate was subsequently filtered through 0.22 μm membranes (Millipore) to capture the prokaryotic cells, metagenomic DNA was then extracted by washing and vortexing the membranes in phosphate buffered saline with Tween20 (2%) before enzymatic lysis (Meta-G-Nome DNA isolation kit; Epicentre). Assessment of DNA quality and concentration was made by TBE agarose (2%) gel electrophoresis and spectrophotometry (Nanodrop ND-1000; ThermoScientific). For each sample, 2 μg of DNA was used to generate Illumina paired-end libraries that were sequenced using an Illumina HiSeq2500. A full description of the metagenomic samples used in this study is available in the supplemental material (Table S1.)

Bioinformatic analyses

Identification of ARGs

ARGs were identified using the Search Engine for Antimicrobial Resistance (SEAR) (Rowe *et al.* 2015). In brief, the pipeline quality checks and filters metagenomic reads, clusters the filtered reads to the ARG-annot (Gupta *et al.* 2014) database of horizontally acquired ARGs and uses the resulting clusters to map the reads and generate a consensus sequence for each ARG in the query metagenome.

Consensus sequences are then aligned to online databases (NCBI genbank, RAC, ARDB), annotated and given an abundance value based on the Reads Per Kilobase per Million (RPKM) value from the read-mapping stage. A full description of SEAR is available in supplemental methods.

Identification of MGEs

MGEs were identified by mapping metagenomic reads to a custom MGE database using Burrows-Wheeler Aligner-mem (default options) (Li & Durbin 2009). The MGE database was built from the NCBI Refseq plasmid genomes dataset, combined with the representative sequences generated from clustering the Integrall dataset (Moura *et al.* 2009) at 97% identity using USEARCH (Edgar 2010). MGE mapping results with less than 90% coverage of the reference sequence were discarded from the analysis. Successfully mapped sequences where then binned into class I and class II integrons, transposons and mobilisable plasmids.

Abundance analysis

The ARG and MGE abundance data was normalised to the number of 16S rRNA sequences as in Bengtsson-Palme *et al.* (2014). In brief, bacterial 16S rRNA sequences were extracted from each metagenome using Metaxa 2.0 (Bengtsson-Palme *et al.* 2015) using default settings and then grafted to sequences from the SILVA RNA database using Megraft (Bengtsson *et al.* 2012) and subsequently clustered using USEARCH (Edgar 2010). ARG abundance values were normalised to 16S sequences by dividing the number of extracted 16S sequences by the length of the 16S gene (Bengtsson-Palme *et al.* 2014).

Taxonomic profiling and pathogen detection

Taxonomic profiling of metagenomes was carried out by mapping sequencing reads to clade-specific marker genes using the Metaphlan package (Segata *et al.* 2012) (default parameters). Metaphlan output was then cross-referenced to the PATRIC database of pathogenic bacteria (Gillespie *et al.* 2011) to annotate potential human-specific bacterial pathogens. Biomarker discovery and identification of differentially abundant features between metagenomes from 2012, 2013 and 2014 was performed using LEfSe (Segata *et al.* 2011). Taxonomic profiling and pathogen data was then combined and presented using the Graphlan package (Segata 2014).

RESULTS

Metagenome analysis

We generated 29.52 Giga base-pairs of data across all samples, with the number of reads produced from the total farm effluent samples being approximately double that produced from the total WWTP effluent samples (Table 1).

Identification of ARGs

In the effluent from the dairy farm we found an average of 7709 reads (0.007%) matching ARGs across the three samples. We found an average of 2740 reads (0.004%) matching ARGs across the three WWTP effluent samples. Only 181 reads (0.0003%) were found to match ARGs from the river source water. A significant diversity of ARGs was observed across the samples, with 53 different ARGs found in total, conferring resistance to seven antimicrobial classes (Figure 1, Table S2). There were 18 ARGs common between the farm and the WWTP effluent samples. The river source water contained the lowest diversity of ARGs (five ARGs, conferring resistance to two antimicrobial classes). When normalised to the number of 16S sequences in each sample, the most abundant ARG across all the samples was found to be *sul2* (sulfonamide resistance) in the WWTP effluent 2014 (0.097 copies per

Table 1 | Summary of the metagenomes generated in the present study

Sample	Read pairs	Gbp	Total ARG reads	% ARGs	% 16S
Farm effluent 2012	44,337,147	4.4337	7,715	0.0087	0.1199
Farm effluent 2013	33,060,321	3.3060	2,317	0.0035	0.0396
Farm effluent 2014	92,074,704	9.2075	13,094	0.0071	0.0775
WWTP effluent 2012	28,696,239	2.8696	4,205	0.0073	0.1086
WWTP effluent 2013	32,980,301	3.2980	250	0.0004	0.0366
WWTP effluent 2014	36636758	3.6637	3,767	0.0051	0.0862
River source water 2014	27,399,641	2.7400	181	0.0003	0.0201

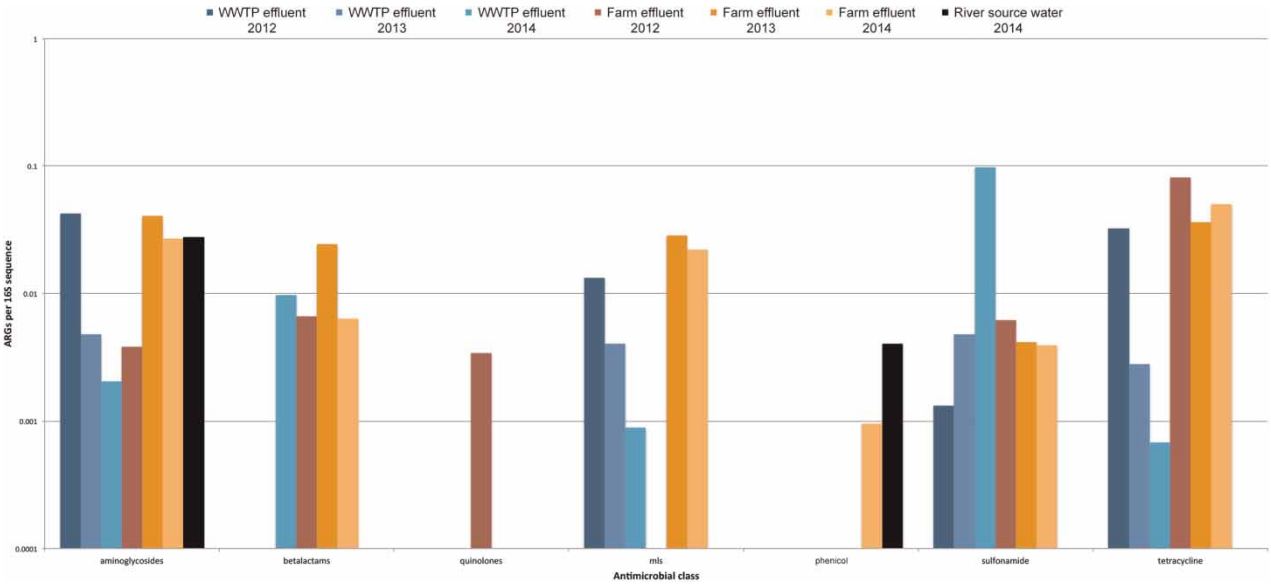

Figure 1 | Abundance of ARGs found in each effluent sample, binned by antimicrobial class. (*Abundance of ARGs is normalised to the number of 16S sequences per sample. The MLS class of antimicrobial represents marcolides, lincosamides and streptogramins*).

16S sequence) and the least abundant ARG was *catB4* (phenicol resistance), found in the farm effluent 2014 (0.0001 copies per 16S sequence). When looking at the effluents individually, tetracycline resistance genes *tetC* (farm effluent 2012) and *tetW* (farm effluent 2013 and 2014) were the most abundant genes within the farm effluent samples. In comparison, the aminoglycoside resistance genes *strA/strB* (WWTP effluent 2012) and the sulfonamide resistance genes *sul1/sul2* (WWTP effluent 2013 and 2014) were the most abundant ARGs within the WWTP effluent samples. On average, the abundance of ARGs in the farm effluents was three times that of the river source water. Similarly, the average abundance of ARGs in the WWTP effluents was double that found in the river source water. In terms of the diversity of ARGs relative to the river source water, the farm effluent had an average of five different ARGs for each ARG found in the river source water, whereas the WWTP effluent had different 2 ARGs for each ARG present in the source water.

When comparing samples across the three years that the samples were taken, the abundance of ARGs was found to decrease year on year in the WWTP effluent for all but sulfonamide resistance genes, which were found to increase over time (11% average change in abundance of sulfonamide resistance genes over three years). The largest change over time for the farm effluent was the 10% increase in the abundance of aminoglycoside resistance genes observed between 2012–2013.

Identification of MGEs

In conjunction with determining the abundance and diversity of ARGs, the effluents were also interrogated for MGEs (Figure 2, Table S3). No MGEs were found to be present in the river source water. Mobilisable plasmids were the most abundant class of MGE found out of the combined metagenomic datasets, although no mobilisable plasmids were identified in the WWTP effluent 2012 or farm effluent 2014 samples. Class I and class II integrons, as well as transposon sequences, were found in all effluent samples. Class I integrons were more abundant in the collective farm effluent samples, compared to class II integrons that were more abundant in the collective WWTP effluent samples.

Taxonomic profiling and pathogen detection

Finally, the effluent metagenomes were subjected to taxonomic profiling. At genus level, the most abundant prokaryotes in the farm samples were *Pseudomonas* (farm effluent 2012) and *Butyrivibrio* (farm effluent 2013 and 2014). The most abundant prokaryotes at genus level in the WWTP samples were *Acinetobacter* (WWTP effluent 2012), *Thiomonas* (WWTP effluent 2013) and *Proteus* (WWTP effluent 2014). For the river source water, the most abundant prokaryotic genus was *Sphingobium*. After cross-referencing the identified species level, clade-specific marker genes for all the metagenomes to the PATRIC

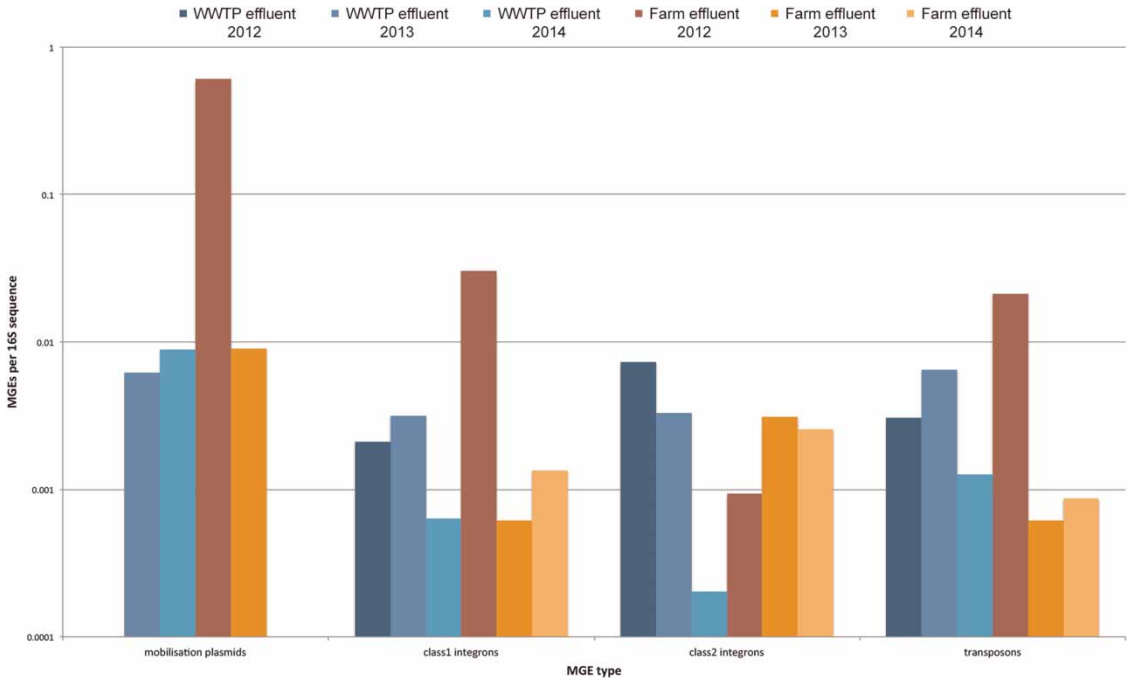

Figure 2 | Abundance of MGEs found in each effluent sample, binned by MGE type. (*Plasmids were binned as mobilisation plasmids if they contained conjugation genes (tra, mob etc.) and integrons were binned as class I or II depending on the Integrall annotation. Relative abundance of MGEs is normalised to the number of 16S sequences per sample).*

pathogen database, a total of 35 species of potential bacterial pathogens were identified (Figure 3, Table S4). The most commonly identified species were *Escherichia coli*, *Arcobacter butzleri*, *Eubacterium rectale*, *Ruminococcus bromii* and *Salmonella enterica*. The WWTP effluent 2014 contained the greatest diversity of potential bacterial pathogens, whereas the river source water and the WWTP effluent 2012 were found to contain the lowest diversity.

DISCUSSION

Through the use of a comparative metagenomic approach, we have shown that two types of effluent entering a shared river catchment contain ARGs and MGEs at higher average abundances than in a background sample of the river source water. This would suggest that effluents such as these are likely to serve as sources of ARGs and thus contribute to the environmental resistome of river catchments and other aquatic environments. It may be appropriate to routinely monitor such effluents as sources of ARGs, particularly when considering the current view of ARGs as environmental contaminants (Pruden *et al.* 2006) and the call for an environmental framework to tackle antimicrobial resistance (Berendonk *et al.* 2015).

One such reason for the high abundance of ARGs in effluents may be the presence of antimicrobial compounds that could consequently provide a selective pressure for the maintenance of ARGs. There have been several studies that document the presence of antimicrobial compounds, from both human and veterinary medicine, in the environment (Kemper 2008; Hu *et al.* 2010). Although these compounds are often present at relatively low concentrations, some studies have shown therapeutic concentrations of antimicrobials being discharged into the environment, such as the effluent from Indian drug manufacturers containing therapeutic concentrations of antimicrobial compounds (Larsson *et al.* 2007). Subsequent studies by Larsson *et al.* found a high abundance of ARGs downstream of the effluent discharge point relative to upstream of the manufacturers and when compared to a Swedish WWTP (Kristiansson *et al.* 2011). While the environmental release of antimicrobial compounds at therapeutic concentrations is largely prevented in the UK, Europe and US through proper wastewater management and controls, clinically important antimicrobials can be found in the environment at sub-inhibitory concentrations and it is possible that these very low antimicrobial concentrations could be enriching for resistant bacteria and promote increased persistence of ARGs (Gullberg *et al.* 2011). Thus, it may be pertinent to couple future environmental ARG monitoring

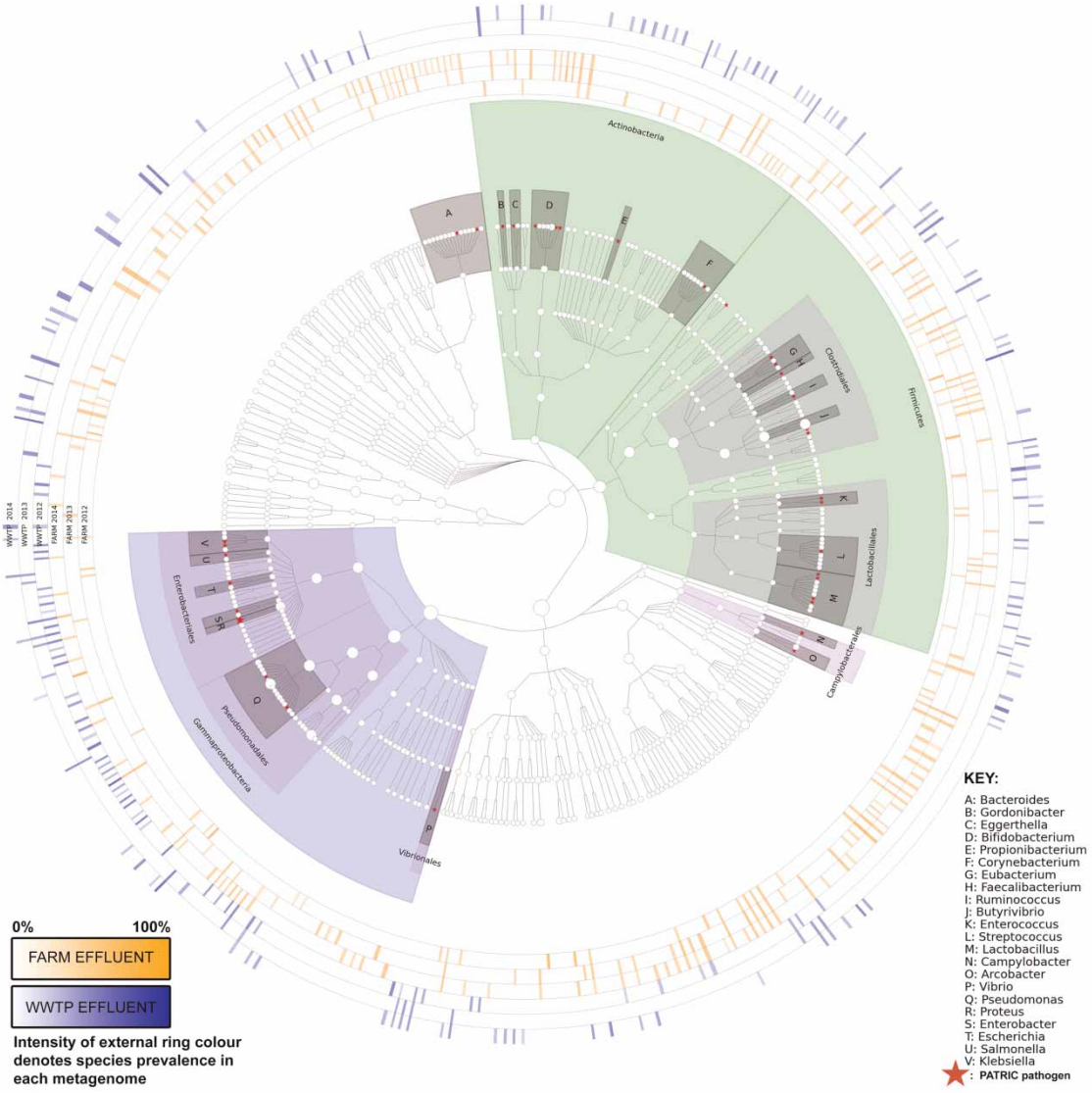

Figure 3 | Metagenomic phylogenetic analysis and annotation of potential bacterial pathogens. (*The phylogenetic tree was built using Graphlan from the merged Metaphlan and LEfSe output for the effluent metagenomes. The PATRIC pathogens are highlighted as red stars and the external rings denote species prevalence in each metagenome).*

studies and risk assessments with information on antimicrobial usage and the antimicrobial concentrations in the effluents being investigated.

Interestingly, the average abundance of ARGs was found to be greater in the farm effluents than in the WWTP effluents (Figure 1). Although these two effluents are from differently treated faecal sources, one being a treated effluent (sedimentation treatment) from a municipal WWTP (i.e. predominantly human faecal source) and the other being an untreated effluent from a farm (predominantly bovine faecal source), this finding does offer some insight into the debate surrounding the relative impact of human and animal contributions to the development of antimicrobial resistance (Phillips *et al.* 2004; Mather *et al.* 2013). The fact that WWTP effluent had undergone a form of water treatment prior to being released into the river catchment, whereas the farm effluent did not, may suggest that some form of water treatment could reduce the abundance or diversity of ARGs. A comparison of WWTP crude influent to the effluent could elaborate on the effectiveness of sedimentation treatment on the abundance of ARGs. Studies have shown that wastewater treatment processes do not completely remove ARGs (Wang *et al.* 2015) and that some WWTP processing can result in an increase in the proportion of antimicrobial resistant bacteria in WWTP effluents (Harris *et al.* 2012). Considering that

effluents may also disseminate antimicrobial compounds, it raises the question as to whether the combination of ARGs and antimicrobial compounds within effluents is resulting in the expression of ARGs and the occurrence of phenotypic antimicrobial resistance. This should be addressed in future studies that aim to assess the risk of ARGs entering the environment.

In terms of the mobility of genes within the effluents, an array of mobilisable plasmids, integrons and transposons were present in the metagenomes (Figure 2) and many of the ARGs identified aligned to the repository of antibiotic resistance cassettes (RAC) (Tsafnat *et al.* 2011). This raises the possibility that the ARGs within the effluents could be readily mobilised into other bacteria, including both directly into pathogens also discharged into the environment and environmental bacteria. These environmental bacteria in turn could pose a risk as potential bacterial intermediaries, harbouring these ARGs in the environment prior to transferring them into other pathogens.

Based on the observations in this study, it is recommended that future Risk Assessments should incorporate direct MGE and pathogen detection with metagenomic assessments of effluents entering river catchments, especially considering the absence of MGEs and the lower diversity of pathogens found in the river source water. This study also showed that a large amount of variation can occur between samples from the same sampling site, possibly as a result of seasonal variation or other environmental factor related to sample collection. It would be benifical to future environmental risk assessments if the impact of seasonal variation on ARG abundance could be determined.

We did however find five resistance genes in the river source water conferring resistance to two classes of antimicrobials. When normalised to 16S sequences the river source water was found to be accountable for the most abundant phenicol resistance gene and the third most abundant aminoglycoside resistance genes out of all the metagenome libraries examined. However, when using the raw SEAR abundance metric, that does not include normalisation to the 16S sequences within the sample, the relative abundance of ARGs from the river source water are reduced relative to the other effluent samples. This raises the question as to whether 16S normalisation is the most appropriate approach to metagenomic abundance estimates as factors such as variation in 16S copy number can skew the data generated as well as interpretation (Case *et al.* 2007). An alternative could be to use the RPKM value generated as part of the SEAR analysis and featured in Table S2.

The metagenomic approach used was relatively less sensitive than more direct-targeted measures of known ARG abundance (e.g. qPCR-based detections (LaPara *et al.* 2011)). The lack of sample replication at each time point also made comparisons between effluents less certain. However the approach had the advantages that it was relatively unbiased and semi-quantitative, giving a good estimation of relative key ARG and MGE abundance and diversity across bacterial populations. It was also potentially able to detect novel ARGs that would otherwise not be found using these more targeted approaches.

CONCLUSION

We have presented a detailed metagenomic analysis of effluents entering a river catchment. Effluents were found to contain an array of ARGs, MGEs and pathogenic bacteria that, when compared to a background sample of the river source water, were found to be more diverse and abundant than in the river source water. This study has shown that the discharge of effluents into river catchments contributes to the dissemination of ARGs, MGEs and pathogenic bacteria, and may play an important role in the propagation of environmental reservoirs of ARGs.

ACKNOWLEDGEMENTS

This research was funded by the Centre for Environment, Fisheries and Aquaculture Science, GlaxoSmithKline and the Biotechnology and Biological Sciences Research Council. Metagenomic sequencing was performed by the Sequencing Service and Wellcome Trust Biomedical Informatics Hub, Exeter (UK).

REFERENCES

Bengtsson, J., Hartmann, M., Unterseher, M., Vaishampayan, P., Abarenkov, K., Durso, L., Bik, E. M., Garey, J. R., Eriksson, K. M. & Nilsson, R. H. 2012 Megraft: a software package to graft ribosomal small subunit (16S/18S) fragments onto full-length sequences for accurate species richness and sequencing depth analysis in pyrosequencing-length metagenomes and similar environmental datasets. *Research in Microbiology* **163** (6–7), 407–412.

Bengtsson-Palme, J., Boulund, F., Fick, J., Kristiansson, E. & Larsson, J. 2014 Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. Frontiers in Microbiology 5.

Bengtsson-Palme, J., Hartmann, M., Eriksson, K. M., Pal, C., Thorell, K., Larsson, D. G. & Nilsson, R. H. 2015 metaxa2: improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. Mol Ecol Resour.

Berendonk, T. U., Manaia, C. M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., Burgmann, H., Sorum, H., Norstrom, M., Pons, M.-N., Kreuzinger, N., Huovinen, P., Stefani, S., Schwartz, T., Kisand, V., Baquero, F. & Martinez, J. L. 2015 Tackling antibiotic resistance: the environmental framework. Nat Rev Micro advance online publication.

Case, R. J., Boucher, Y., Dahllöf, I., Holmström, C., Doolittle, W. F. & Kjelleberg, S. 2007 Use of 16S rRNA and rpoB Genes as molecular markers for microbial ecology studies. *Applied And Environmental Microbiology* **73** (1), 278–288.

Chen, B., Yang, Y., Liang, X., Yu, K., Zhang, T. & Li, X. 2013 Metagenomic profiles of antibiotic resistance genes (ARGs) between human impacted estuary and deep ocean sediments. *Environmental Science & Technology* **47** (22), 12753–12760.

D'Costa, V. M., McGrann, K. M., Hughes, D. W. & Wright, G. D. 2006 Sampling the antibiotic resistome. *Science* **311** (5759), 374–377.

Dancer, D., Baker-Austin, C., Lowther, J. A., Hartnell, R. E., Lees, D. N. & Roberts, L. O. 2014 Development and integration of quantitative real-time PCR methods for detection of mitochondrial DNA and methanobrevibacter smithii nifH gene as novel microbial source tracking tools. *Environmental Forensics* **15** (3), 256–264.

Dantas, G., Sommer, M. O., Oluwasegun, R. D. & Church, G. M. 2008 Bacteria subsisting on antibiotics. *Science* **320** (5872), 100–103.

Davies, J. & Davies, D. 2010 Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews: MMBR* **74** (3), 417–433.

Devarajan, N., Laffite, A., Graham, N. D., Meijer, M., Prabakar, K., Mubedi, J. I., Elongo, V., Mpiana, P. T., Ibelings, B. W., Wildi, W. & Pote, J. 2015 Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in central Europe. *Environmental Science & Technology* **49** (11), 6528–6537.

Edgar, R. C. 2010 Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26** (19), 2460–2461.

Forsberg, K. J., Reyes, A., Wang, B., Selleck, E. M., Sommer, M. O. A. & Dantas, G. 2012 The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337** (6098), 1107–1111.

Gillespie, J. J., Wattam, A. R., Cammer, S. A., Gabbard, J. L., Shukla, M. P., Dalay, O., Driscoll, T., Hix, D., Mane, S. P., Mao, C., Nordberg, E. K., Scott, M., Schulman, J. R., Snyder, E. E., Sullivan, D. E., Wang, C., Warren, A., Williams, K. P., Xue, T., Seung Yoo, H., Zhang, C., Zhang, Y., Will, R., Kenyon, R. W. & Sobral, B. W. 2011 PATRIC: the comprehensive bacterial bioinformatics resource with a focus on human pathogenic species. *Infection and Immunity* **79** (11), 4286–4298.

Gillings, M. R. 2013 Evolutionary consequences of antibiotic use for the resistome, mobilome and microbial pangenome. Frontiers in Microbiology 4.

Gullberg, E., Cao, S., Berg, O. G., Ilbäck, C., Sandegren, L., Hughes, D. & Andersson, D. I. 2011 Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* **7** (7), e1002158.

Gupta, S. K., Padmanabhan, B. R., Diene, S. M., Lopez-Rojas, R., Kempf, M., Landraud, L. & Rolain, J.-M. 2014 ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrobial Agents and Chemotherapy* **58** (1), 212–220.

Harris, S., Cormican, M. & Cummins, E. 2012 The effect of conventional wastewater treatment on the levels of antimicrobial-resistant bacteria in effluent: a meta-analysis of current studies. *Environmental Geochemistry and Health* **34** (6), 749–762.

Hu, X., Zhou, Q. & Luo, Y. 2010 Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China. *Environmental Pollution* **158** (9), 2992–2998.

Kemper, N. 2008 Veterinary antibiotics in the aquatic and terrestrial environment. *Ecological Indicators* **8** (1), 1–13.

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H. & Larsson, D. G. J. 2011 Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PloS one* **6** (2), e17038.

LaPara, T. M., Burch, T. R., McNamara, P. J., Tan, D. T., Yan, M. & Eichmiller, J. J. 2011 Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into duluth-superior harbor. *Environmental Science & Technology* **45** (22), 9543–9549.

Larsson, D. G., de Pedro, C. & Paxeus, N. 2007 Effluent from drug manufactures contains extremely high levels of pharmaceuticals. *J Hazard Mater* **148** (3), 751–755.

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K. M., Wertheim, H. F. L., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., Kariuki, S., Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D. & Cars, O. 2013 Antibiotic resistance – the need for global solutions. *The Lancet Infectious Diseases* **13** (12), 1057–1098.

Levy, S. B. & Marshall, B. 2004 Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine* **10** (12 Suppl), S122–S129.

Li, H. & Durbin, R. 2009 Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25** (14), 1754–1760.

Li, J., Wang, T., Shao, B., Shen, J., Wang, S. & Wu, Y. 2012 Plasmid-mediated quinolone resistance genes and antibiotic residues in wastewater and soil adjacent to swine feedlots: potential transfer to agricultural lands. *Environmental Health Perspectives* **120** (8), 1144–1149.

Li, J., Cheng, W., Xu, L., Strong, P. J. & Chen, H. 2015 Antibiotic-resistant genes and antibiotic-resistant bacteria in the effluent of urban residential areas, hospitals, and a municipal wastewater treatment plant system. *Environmental Science and Pollution Research* **22** (6), 4587–4596.

Linares, J. F., Gustafsson, I., Baquero, F. & Martinez, J. L. 2006 Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences of the United States of America* **103** (51), 19484–19489.

Lu, Z., Na, G., Gao, H., Wang, L., Bao, C. & Yao, Z. 2015 Fate of sulfonamide resistance genes in estuary environment and effect of anthropogenic activities. *Science Total Environmental* **527–528C**, 429–438.

Mather, A. E., Reid, S. W. J., Maskell, D. J., Parkhill, J., Fookes, M. C., Harris, S. R., Brown, D. J., Coia, J. E., Mulvey, M. R., Gilmour, M. W., Petrovska, L., de Pinna, E., Kuroda, M., Akiba, M., Izumiya, H., Connor, T. R., Suchard, M. A., Lemey, P., Mellor, D. J., Haydon, D. T. & Thomson, N. R. 2013 Distinguishable Epidemics of Multidrug-Resistant Salmonella Typhimurium DT104 in Different Hosts. *Science* **341** (6153), 1514–1517.

Moura, A., Soares, M., Pereira, C., Leitao, N., Henriques, I. & Correia, A. N. 2009 INTEGRALL: a database and search engine for integrons, integrases and gene cassettes. *Bioinformatics* **25** (8), 1096–1098.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R. & Waddell, J. 2004 Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *The Journal of Antimicrobial Chemotherapy* **53** (1), 28–52.

Port, J. A., Cullen, A. C., Wallace, J. C., Smith, M. N. & Faustman, E. M. 2014 Metagenomic frameworks for monitoring antibiotic resistance in aquatic environments. *Environmental Health Perspectives* **122** (3), 222–228.

Pruden, A., Pei, R., Storteboom, H. & Carlson, K. H. 2006 Antibiotic resistance genes as emerging contaminants: studies in northern Colorado. *Environmental Science and Technology* **40** (23), 7445–7450.

Rowe, W., Baker, K. S., Verner-Jeffreys, D., Baker-Austin, C., Ryan, J. J., Maskell, D. & Pearce, G. 2015 Search engine for antimicrobial resistance: a cloud compatible pipeline and web interface for rapidly detecting antimicrobial resistance genes directly from sequence data. *PloS one* **10** (7), e0133492.

Segata, N. 2014 GraPhlAn, https://bitbucket.org/nsegata/graphlan/wiki/Home.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. & Huttenhower, C. 2011 Metagenomic biomarker discovery and explanation. *Genome Biology* **12** (6), R60.

Segata, N., Waldron, L., Ballarini, A., Narasimhan, V., Jousson, O. & Huttenhower, C. 2012 Metagenomic microbial community profiling using unique clade-specific marker genes. *Nature Methods* **9** (8), 811–814.

Tao, R., Ying, G. G., Su, H. C., Zhou, H. W. & Sidhu, J. P. 2010 Detection of antibiotic resistance and tetracycline resistance genes in Enterobacteriaceae isolated from the Pearl rivers in South China. *Environmental Pollution* **158** (6), 2101–2109.

Tsafnat, G., Copty, J. & Partridge, S. R. 2011 RAC: Repository of antibiotic resistance cassettes. *Database* **2011**.

Wang, J., Mao, D., Mu, Q. & Luo, Y. 2015 Fate and proliferation of typical antibiotic resistance genes in five full-scale pharmaceutical wastewater treatment plants. *Science Total Environmental* **526**, 366–373.

Wellington, E. M. H., Boxall, A. B. A., Cross, P., Feil, E. J., Gaze, W. H., Hawkey, P. M., Johnson-Rollings, A. S., Jones, D. L., Lee, N. M., Otten, W., Thomas, C. M. & Williams, A. P. 2013 The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *The Lancet Infectious Diseases* **13** (2), 155–165.

WHO 2012 The evolving threat of antimicrobial resistance–options for action. WHO (ed.), Geneva.

William, H. G., Stephen, M. K., Larsson, D. G. J., Xian-Zhi, L., Joseph, A. R., Pascal, S., Kornelia, S., Mohammed, T., Ed, T., Elizabeth, M. W., Gerard, D. W. & Yong-Guan, Z. 2013 Influence of humans on evolution and mobilization of environmental antibiotic resistome. *Emerging Infectious Disease Journal* **19** (7).

Wright, G. D. 2007 The antibiotic resistome: the nexus of chemical and genetic diversity. Nature reviews. *Microbiology* **5** (3), 175–186.

Zhang, X. X. & Zhang, T. 2011 Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. *Environmental Science & Technology* **45** (7), 2598–2604.

Zhang, X. X., Zhang, T. & Fang, H. H. 2009 Antibiotic resistance genes in water environment. *Applied Microbiology and Biotechnology* **82** (3), 397–414.

First received 10 July 2015; accepted in revised form 1 December 2015. Available online 18 December 2015